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(57) Abstract

Living bacteria are preserved for use in human and veterinary medicine by mixing them with trehalose and/or glycerol and drying the mixture.

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MEDICAL PREPARATIONS

This invention relates to a method of preserving bacteria for use in human and veterinary medicine, and to medical preparations containing them.

Various bacteria are used in human and veterinary medicine, especially in the production of vaccines. Live bacterial vaccines containing living bacterial cells are used both in human medicine and veterinary medicine for the prevention of disease. To be effective, a minimum dose of live bacterial cells is usually required. A major problem in the use of live bacterial vaccines is in preserving the live bacteria in the period between their production and their administration. In many cases, special precautions must be taken to maintain the organism viable and/or to maintain the minimum activity required for the vaccine to be effective. The problems of maintaining viability can impose severe limitations on the use of these materials.

WO 87/00196 describes a method of protecting proteins and other biological macromolecules against denaturation during drying, in which method an aqueous system containing the macromolecule is dried above its freezing point in the presence of trehalose. The method is described as useful for making dried vaccines containing killed microorganisms. WO 89/06542 describes a method of preserving live viruses by drying an aqueous system comprising the live virus in the frozen state or at ambient temperature in the presence of trehalose. The live viruses

can be used, intact or attenuated, as immunogenic vaccines.

Neither of these patent specifications suggests or discloses any way of preserving living bacteria, either for use in vaccines or for any other purpose. Indeed, the concept of preserving complex organisms such as live bacteria is very different from that of preserving either inanimate macromolecules or simple viruses. Furthermore, in the case of live bacteria for use in vaccines, any preservation treatment must be such that the immunogenic characteristics of the bacteria are not deleteriously affected and no regression or other undesirable change brought about.

We have now found that bacteria useful in human and veterinary medicine can be treated to extend their life. In particular we have found that if these bacteria are dried in the presence of trehalose, and/or glycerol, the dried bacteria can be stored for extended periods, at a broad range of temperatures, with greatly improved viability. Furthermore, it appears that the vitally important immunogenic properties can be preserved and there is no increase in the tendency to regression.

According to one aspect of the present invention, therefore, there is provided a method of preserving live bacteria for use in human and veterinary medicine, wherein the bacteria are mixed with trehalose and/or glycerol, and the mixture is then dried. Upon reconstitution, with water or other aqueous fluid, live bacterial suspensions are obtained, the extent of viability being dependent largely upon the particular organism concerned.

The invention also includes a live bacterial vaccine for human or veterinary use, which comprises live bacteria which have been preserved by the method of the invention.

The present invention is applicable to a wide variety of bacteria, and especially those bacteria which are of use in human and veterinary medicine. Among these are, for example, the Vibrio cholerae, many Enterobacteracea such

as the Shigella species including for example Shigella sonnei and Shigella flexneri, the Salmonella species including for example Salmonella typhi TY21a, campylobacter, Lactococcus, the Staphylocci and other gram positive bacteria. These bacteria may be used as such, or they may be used as vectors to carry genetic material which can then express itself after entry to the body and thereby induce protective immune responses. Examples are those for the hepatitis B genome, or the ß-subunit of cholera toxin known to be useful in providing protection against Entero Toxin producing E.coli whcih are mainly responsible for travellers' diarrhoea. Live bacteria may also be used to simply carry immunogenic material for presentation to the body's immune mechanisms rather than requiring their expresion through multiplication of the carrying bacteria in the gut lumen or body tissue.

It is an important feature of the present invention that, in many cases, the live bacteria dried in accordance with the invention can be stored under ambient conditions and no special precautions (eg. low temperature) are necessary. This is a very significant advantage since vaccines (in dry form) can thus be stored at room temperature over an extended period of time. One example of this is in veterinary medicine where, hitherto, certain pig vaccines have had a limited life such that they have had to be added to the animal feed only at the time of consumption. Live bacterial vaccines of the present invention for this purpose can be mixed into the dry feed at the time of feed manufacture and will remain viable over a reasonable time until the feed is given to the animal.

In the method of the invention, various drying procedures may be used provided they are per se suitable for drying bacterial systems without damage to the bacteria. For commercial purposes, drying under a vacuum with or without freezing will usually be necessary but for small scale operation room temperature-atmospheric pressure

evaporation drying can be used. We prefer to dry at room temperature under a vacuum, eg. about 25 millibars. When the liquid has been removed to leave a solid residue, we prefer then to maintain the vacuum for a further period, eg. 12 hours, and then to pack the dried cells (eg. by sealing them in ampoules or plastic pots) whilst maintaining the vacuum. Alternatively, the dried material may be hermetically sealed at room temperature and atmosphere pressure.

Freeze drying can alternatively be used but we have not found it to be quite so effective in preserving viability. It is possible to use the freeze drying technique described in WO88/04551, wherein the material to be dried is foamed with a gas, to form a freeze dried foam with the bacterial cells dispersed therethrough. Reference should be made to International Publication WO 88/04551 for further details. In this case, drying is best carried out in the final plastic packaging container, and the container then heat sealed with lacquer-coated metal foil provided that such plastic packaging material has the highest possible moisture barrier properties.

As will be well understood, bacterial cultures are grown in aqueous suspensions. Before treating the bacteria with the trehalose or glycerol according to the invention, they are preferably separated from the nutrient medium in which they have been grown. This separation can be effected in any convenient way, such as by centrifuging or, in some cases, by sedimentation for example. The separated bacterial cells are then mixed with the trehalose and/or glycerol. Usually, this is done by dissolving the trehalose or glycerol in water and then adding the cells thereto. It is possible, however, if desired, to avoid the use of aqueous suspensions by dissolving the trehalose and/or glycerol in a suitable non-aqueous solvent, or by using glycerol itself (with or without dissolved trehalose) as the suspending medium for the bacterial cells.

When solutions of trehalose and/or glycerol are used, the optimum concentration will depend on the particular bacterium used. The preferred amount of each of these materials is generally from about 5% to about 20% by weight of the bacteria-containing system, but greater amounts eg. up to 40% or more may sometimes be useful. Either trehalose or glycerol can be used alone, or a mixture of the two can be used.

In the method of the invention, the aqueous (or other) system in which the bacteria are suspended for drying can include other materials (in addition to the trehalose and/or glycerol) as desired. For example, it may be advantageous to include non-toxic water excluders, such as sodium sulphate, potassium chloride or calcium lactate. The use of these materials is described in International Publication no. WO 89/06976 to which reference should be made for further details.

Whilst the present invention is broadly applicable to many bacteria, it is particularly useful for the preservation of various Salmonella mutant strains used in typhoid vaccines. These strains are described in British patent no. 1291214 and, currently, the most interesting commercially available strain is Salmonella typhi TY21a. This particular strain is widely used in live bacterial vaccines against typhoid. However, whilst the strain is very effective for this purpose, as a practical matter it is difficult to produce in high yields and it is not very stable. By the method of the present invention, its stability when stored can be markedly improved. This strain is also of interest as a potential carrier of other materials.

Live bacteria such as S. typhi TY21a dried in accordance with the invention can be formulated for use as vaccines. This is preferably effected immediately prior to their administration by resuspending the bacterial cells in water, possibly together with an antacid and/or other

materials, for oral ingestion.

In order that the invention may be more fully understood, the following Examples are given by way of illustration only:

Example 1

An aqueous suspension of S.typhi TY2Ia was made containing 2.3×10^{10} viable organisms per ml. Various quantities of trehalose and/or glycerol were added to samples of the suspension, as indicated below. The suspensions were then coated on to filter paper and air-dried at room temperature. Live bacterial counts were made (a) at the time of coating the paper, and (b) after the periods of time indicated. As can be seen, upon initial drying the count fell markedly, but stabilised. By way of comparison, a sample of the original suspension was also dried (without trehalose) in the same way. Its counts are also shown.

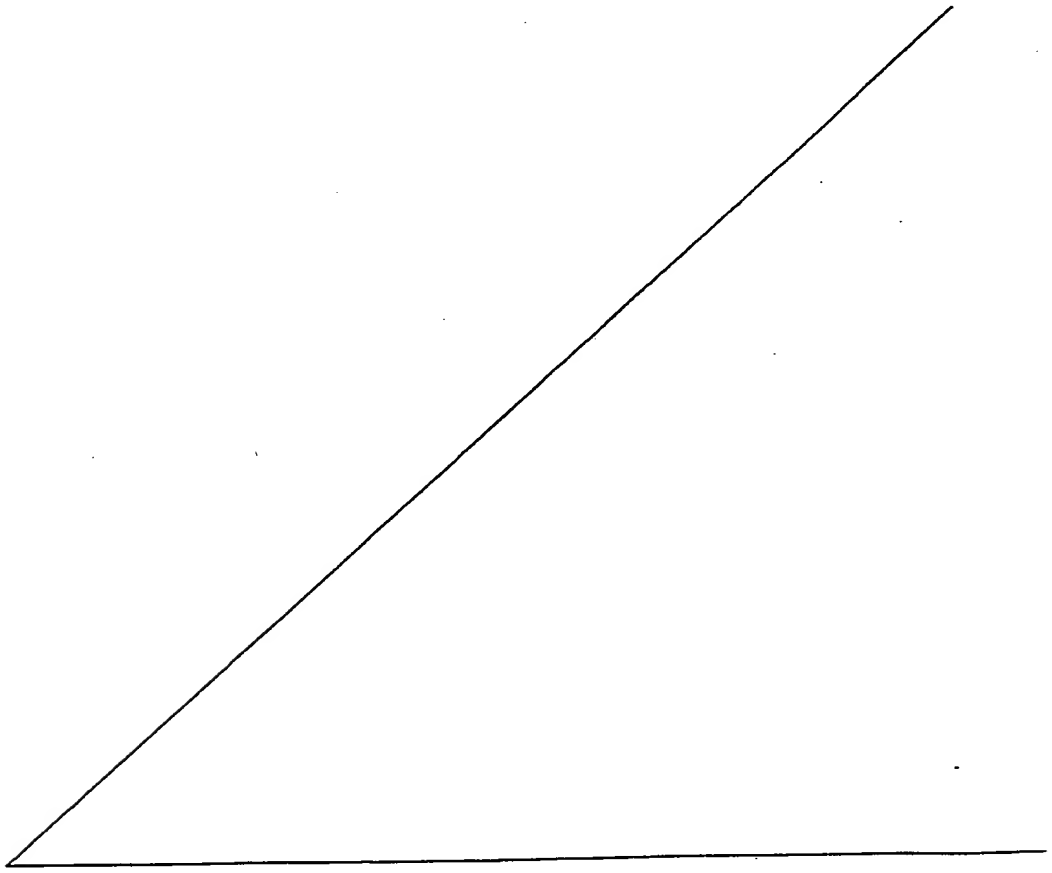

TABLE 1

Time after drying	% Trehalose 0	20%
24 hrs	7×10^4	7×10^7
48 hrs	0	1.8×10^7
7 days	0	1×10^6

Initial count of suspension before application to filter paper: 2×10^{10}

TABLE II

Time after drying	20% glycerol	10% glycerol	10% glycerol 20% trehalose mixture	20% glycerol 10% trehalose mixture	20% glycerol 20% trehalose mixture
24 hrs	1×10^6	4×10^6	6×10^6	2.5×10^6	2.5×10^6
48 hrs	1.5×10^5	2×10^6	3.5×10^6	1.1×10^6	2×10^6

Initial count of suspension before application to filter paper: 5×10^8

Example 2

Example 1 was repeated using freeze drying. The results obtained were:

TABLE III

Time after drying	% Trehalose 0	20%
24 hrs	5.4×10^7	1×10^6
7 days	0	3×10^5

Example 3

A vaccine containing the preserved product of Example 2 comprises a gastric acid resistant capsule containing freeze dried powder of S. Typhi TY21a in an amount to provide a viable count of a minimum of 10^9, ideally from 1 to 5 x 10^{10}.

An alternative vaccine formulation comprises an aqueous suspension of the freeze dried powder of S. Typhi TY21a according to the invention, providing a viable count of 10^9, ideally from 1 to 5 x 10^{10}. This would normally be administered in conjunction with a gastric acid buffer.

Example 4

S. typhi TY21a organisms were cultured overnight. The culture fluid was aliquoted into 6 ml portions and centrifuged for 10 minutes at 3,000 rpm. The supernatant was decanted and 6 ml of either 20 or 40% trehalose was added. This mixture was then vortexed and left to stand for two hours. It was then dispensed in 25 ml aliquots in

sterile ampoules. It was spun in a freeze dryer with no pre-freezing for 30 minutes and then vacuum-dried for three hours. It was dried overnight for 15 hours and sealed under vacuum. Viable counts were performed at times Oh and 24h, as well as at days 7 and 14. Ampoules were stored at either room temperature or 4°C. The results are shown in Table IV.

TABLE IV

Trehalose	T_0 4°C	T_{24} 4°C	T_7 4°C	T_{14} 4°C
0%	1×10^8	1×10^4	0	0
	1×10^8			
20%	1×10^9	1×10^7	5×10^7	3×10^7
	9×10^8	5×10^6	5×10^7	5×10^7
40%	7×10^8	2×10^7	1.1×10^8	5×10^7
	1×10^9	4×10^7	3×10^8	3.8×10^7

EXAMPLE 5

Various organisms (see Table V) were incubated overnight in brain/heart infusion, centrifuged and resuspended in 20% trehalose. One-hundred microlitres of this solution was inoculated onto sterile filter paper discs and air-dried. Viability was checked at times Oh and 24h, and at days 7 and 14. The filter papers were reconstituted by placing the filter paper discs into a universal container in 1 ml of sterile distilled water and shaking vigorously for a few

minutes to break up the filter paper. The results are shown in Table V.

TABLE V

Organism		T_0 4°C	T_{24} 4°C	T_7 4°C	T_{14} 4°C
S.aureus	1	1×10^{10}	-	1.5×10^{8}	5×10^{7}
"	2	1×10^{10}	-	1.1×10^{6}	3×10^{5}
"	3	1×10^{10}	-	5×10^{7}	5×10^{5}
"	4	1×10^{10}	5×10^{7}	1.5×10^{7}	1.7×10^{6}
"	5	1×10^{10}	3.5×10^{8}	1×10^{7}	5×10^{7}
S.epidermidis	1	1×10^{10}	-	1.5×10^{8}	5×10^{7}
"	2	1×10^{10}	-	4×10^{6}	2×10^{6}
"	3	7.5×10^{9}	-	1×10^{6}	3×10^{6}
"	4	1×10^{10}	2×10^{7}	1×10^{7}	5×10^{6}
"	5	1×10^{10}	1×10^{7}	1×10^{7}	1×10^{6}
Salmonella	1	1.5×10^{10}	-	7.5×10^{7}	1×10^{5}
"	2	7.5×10^{9}	7.5×10^{5}	1.5×10^{6}	2×10^{6}
"	3	1×10^{10}	-	1.1×10^{6}	2×10^{6}
"	4	1×10^{10}	2×10^{6}	4×10^{4}	5×10^{5}
"	5	3.2×10^{9}	2×10^{6}	1×10^{6}	1×10^{7}
Streptococcus pyogenes	1	3×10^{8}	5×10^{7}	5×10^{6}	5×10^{5}
	2	7×10^{8}	5×10^{7}	5×10^{7}	-
	3	2×10^{8}	5×10^{7}	5×10^{6}	3×10^{5}
	4	1×10^{9}	5×10^{4}	4×10^{4}	1×10^{4}
E. coli	1	1×10^{10}	5×10^{8}	3.5×10^{7}	8.5×10^{4}
"	2	7.5×10^{9}	5×10^{8}	2×10^{6}	1.5×10^{6}

TABLE V (contd)

E. coli	3	1×10^{10}	5×10^{8}	1×10^{7}	1.5×10^{6}
"	4	2×10^{9}	1×10^{7}	1.5×10^{5}	1×10^{6}
"	5	1×10^{10}	1.5×10^{8}	2×10^{6}	1×10^{7}

CLAIMS:

1. A method of preserving live bacteria for use in human and veterinary medicine, wherein the bacteria are mixed with trehalose and/or glycerol, and the mixture is then dried.

2. A method according to claim 1, wherein the bacteria are Vibrio cholerae, or from the Shigella, Lactococcus, Salmonella or Staphylococcus species.

3. A method according to claim 2, wherein the bacteria are Salmonella typhi TY21a.

4. A method according to claim 1,2 or 3, wherein the mixture is dried at ambient temperature.

5. A method according to claim 1,2,3 or 4, wherein the mixture is dried under reduced pressure.

6. A method according to claim 5, wherein the dried mixture is hermetically sealed in a container for storage whilst still under vacuum.

7. A method according to any of claims 1 to 4, wherein the mixture is freeze dried.

8. A method according to any preceding claim wherein said mixture comprises an aqueous solution of trehalose and/or glycerol, and the said bacteria.

9. A method according to claim 8, wherein the mixture contains from about 5% to about 20% by weight of trehalose and/or glycerol.

10. A method according to claim 8 or 9, wherein the

mixture also contains a non-toxic water excluder.

11. A method according to claim 7, wherein the mixture comprises a solution of trehalose and/or glycerol with the bacteria suspended therein, and wherein a gas is dispersed throughout the mixture and the mixture dried to form a freeze-dried foam.

12. A method according to claim 11, wherein a unit volume of the mixture is freeze-dried to provide a freeze-dried unit volume of unit dosage of live bacteria.

13. A live bacterial vaccine for human or veterinary use, which comprises live bacteria which have been preserved by the method of any of claims 1 to 12.

14. A container which is hermetically sealed and which contains dry live bacteria preserved by the method of any of claims 1 to 12.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/02243

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N1/04; A61K39/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	DD,C,115 919 (W. ERLER ET AL.) 20 October 1975	1,2,5, 7-9, 13-14
Y	see page 2, column 2, line 20 - line 22; claim; examples	1-9,12

X	GB,A,1 443 392 (W.R. GRACE & CO) 21 July 1976	1,2,5, 7-9,10, 12-14
Y	see claims 1,7-9 see page 2, line 111 - line 124; examples 9,20 see page 24, line 48 - line 72	1,4-9,12

P,X	WO,A,9 208 355 (LIPHATECH INC) 29 May 1992 see claims 1-4 see page 7, line 17 - page 8, line 28	1,4,8-10

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 MARCH 1993	29.03.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	COUCKE A.O.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8 906 542 (QUADRANT BIORESOURCES LIMITED) 27 July 1989 see page 10, paragraph 2; claims	1,4-9,12
Y	GB,A,2 135 190 (HUMAN OLTOANYAGTERMELO ES KUTATO INTEZET) 30 August 1984 see claims; examples 2,3	2,3
Y	Section Ch, Week 7801, Derwent Publications Ltd., London, GB; Class B04, Page 78, AN 01553 & SU,A,549 470 (MOSC VACCINE SERUM) 17 June 1977 see abstract	2,3
P,A	DD,A,299 213 (SÄCHSISCHES SERUMWERK GMBH) 9 April 1992 see abstract; claims see page 1, paragraph 5	1,5,7-9, 13,14

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9202243
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DD-A-299213		None		

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